

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

RAVEN *et al.*

Appl. No.: 10/614,370

Filed: July 8, 2003

For: **Degradation and Detection of TSE
Infectivity**

Confirmation No.: 7846

Art Unit: *To Be Assigned*

Examiner: *To Be Assigned*

Atty. Docket: 1581.0990001/RWE/VSR

**Submission of Certified Copies of 35 U.S.C. § 119(a)-(d)
Priority Documents in Utility Application**

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

Submitted herewith is a certified copy of Applicants' 35 U.S.C. § 119(a)-(d)
priority document, to perfect the claim to priority filed on July 8, 2003.

Country	Priority Document Appl. No.	Filing Date
United Kingdom	0100420.9	January 8, 2001
United Kingdom	0104696.0	February 26, 2001
United Kingdom	0216146.1	July 11, 2002

Prompt acknowledgment of this submission is respectfully requested.

Respectfully submitted,
Sterne, Kessler, Goldstein & Fox p.l.l.c.

Robert W. Esmond
Attorney for Applicants
Registration No. 32,893

Date: December 16, 2003

1100 New York Avenue, N.W.
Washington, D.C. 20005-3934
(202) 371-2600
210277.1





INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

I also certify that the application is now proceeding in the name as identified herein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.



Signed

Dated 3 November 2003



INVESTOR IN PEOPLE

GB 0100420.9

By virtue of a direction given under Section 30 of the Patents Act 1977, the application is proceeding in the name of:

HEALTH PROTECTION AGENCY,
Porton Down,
SALISBURY,
Wiltshire,
SP4 0JG,
United Kingdom

Incorporated in the United Kingdom,

[ADP No. 08638108001]

Patents 1977
(Rule 16)

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)



The Patent Office

Cardiff Road
Newport
Gwent NP9 1RH

1. Your reference

GWS/22516

2. Patent application number

(The Patent

0100420.9

8 JAN 2001

09JAN01 E596292-1 D02000

P01/7700 0.00-0100420.9

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Microbiological Research Authority
CAMR

Porton Down
Salisbury
Wiltshire SP4 0JG

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

UK

656616000

SECTION 48 (107 ACT) APPLICATION FILED 12/8/03

4. Title of the invention

Degradation of TSE and similar agents

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

MATHYS & SQUIRE
100 Gray's Inn Road
London WC1X 8AL
United Kingdom

Patents ADP number (if you know it)

1081001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number
(if you know it)

Date of filing
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

- a) any applicant named in part 3 is not an inventor, or
 - b) there is an inventor who is not named as an applicant, or
 - c) any named applicant is a corporate body.
- See note (d))

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form

Description	15
Claim(s)	5
Abstract	0
Drawing(s)	4

Handwritten signature

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

Request for preliminary examination and search (Patents Form 9/77)

1

Handwritten signature

Request for substantive examination (Patents Form 10/77)

Any other documents (please specify)

11.

I/We request the grant of a patent on the basis of this application.

Signature

Mathys & Squire

Date

MATHYS & SQUIRE

8 January 2001

12. Name and daytime telephone number of person to contact in the United Kingdom

GWS - 020 7830 0000

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

- If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.
- Write your answers in capital letters using black ink or you may type them.
- If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.
- If you have answered 'Yes' Patents Form 7/77 will need to be filed.
- Once you have filled in the form you must remember to sign and date it.
- For details of the fee and ways to pay please contact the Patent Office.

DUPLICATE

DEGRADATION OF TSE AND SIMILAR AGENTS

5 The invention is in the field of methods and compositions for the sterilisation of materials and apparatus that may have been contaminated with infectious agents. In particular, the invention relates to methods for the inactivation of transmissible spongiform encephalopathy (TSE) agents and provides compositions for degrading TSE located on or within infected materials.

10 Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurological diseases that include Creutzfeld-Jacob disease (CJD) and Kuru in humans, bovine spongiform encephalopathy (BSE) in cattle and Scrapie in sheep. TSEs are characterised by the conversion of a normal host protein into a pathogenic protein within the brain tissue of an infected animal. The pathogenic form of protein is often referred to as a prion and is highly
15 resistant to physical and chemical degradation. The prion is believed to be the transmissible agent through which the TSE disease is passed on between animals.

20 There has been considerable public alarm in recent years over the risks associated with consumption of meat products, and especially beef, potentially infected with BSE, the bovine form of TSE. Much of this concern is associated with the belief that the BSE prion when eaten by a human may in some cases cause the incurable human form of the disease, referred to as variant CJD (vCJD). Rigorous practices have been adopted in the agricultural
25 and meat rendering industries to reduce the risk of cross contamination between BSE infected carcasses and meat that is destined for human consumption or other animal derived products such as tallow. However, BSE infected animals can still be unintentionally processed in abattoirs, especially if the animal is in the early stages of the disease and therefore undetected as
30 an infected TSE host. There is also a considerable risk in disposal of known BSE infected material, particularly if the equipment used in the disposal operation is then reused in normal rendering practices without adequate sterilisation.

35 Sterilisation of instruments and equipment following potential exposure to TSE infected tissue is of primary importance. In particular, surgical equipment such as scalpels, forceps and endoscopes should be thoroughly sterilised before use on patients to avoid disease transmission.

It has been reported that the CJD infectious agent was accidentally transferred on surgical electrodes, inserted into the brain of a human patient with CJD, to two other previously uninfected patients (Bernouli et al (1977) The Lancet i: pp478-479). The electrodes concerned were sterilised with ethanol and formaldehyde vapour between each procedure, conditions previously thought to be sufficient to eliminate virtually all infectious agents, and yet the CJD infectious agent was able to withstand such harsh conditions and infect the recipient patients' brain tissue.

TSE transmission is typically observed in cases where infected material is transferred between animals or implanted into an animal. As described previously, incomplete or inadequate sterilisation of surgical instruments can lead to such transfer of infected material between patients. Even the most rigorous chemical cleaning and steam sterilisation procedures can fail to remove blood and tissue from surgical instruments, especially in the jaws or joints of forceps and clamps (Laurenson (1999) The Lancet, 20 Nov). Thus, the risk of unintentional TSE transfer can be unnecessarily high.

TSE agents, or prions, are known to be highly resistant to denaturation and degradation, more so than would normally be expected for a protein. Taylor (J. Hosp. Infect. (1999) 43 supplement, pp S69-76) reviews a number of methods for inactivating prion proteins.

Chemical methods for inactivating TSE prion proteins include treatment of infected material with sodium hydroxide or sodium hypochlorite solutions (Taylor et al. (1994) Arch. Virol. 139, pp313-326), although, infectivity of the prion is shown to survive exposure to 2M sodium hydroxide for up to two hours.

Alternative methods for inactivating TSE prions include autoclaving, but again BSE and scrapie agent has been shown to survive treatment at 134-138°C for 18 minutes (Taylor et al. *ibid*). Thus, a combined chemical/heating approach has been proposed in which infected materials are exposed to 1M sodium hydroxide followed by autoclaving at 121°C for between 30 and 60 minutes (Taylor, J. Hosp. Infect. (1999) 43 supplement, pp S69-76). This combined method has shown that inactivation of TSE infectious agents can be achieved, albeit under very harsh conditions.

However, many materials, such as plastics, polymers and non-protein animal derivatives, cannot be exposed to such extreme conditions without themselves being destroyed. The chemical and physical processes described above are only really suitable for sterilising metal instruments and surgical tools that are not too large in size and which can fit inside a standard autoclave. More delicate instruments such as endoscopes cannot be exposed to extreme conditions of high temperature without the risk of permanent damage to their internal components.

Further, chemical processes typically involve the use of caustic and/or chaotropic agents which are hazardous to handle and dispose of. It would, therefore, be desirable to provide a method for inactivating TSE prions without the need to use large amounts of hazardous substances and which method could be scaled up for use on larger objects and areas as well as on smaller objects.

Taylor (Vet. J. (2000) 159 pp10-17) describes tests using proteolytic enzymes to deactivate prion proteins. Proteases such as trypsin have little effect in non-denaturing conditions (Taylor (2000) p. 14) but other proteases such as proteinase K may have an effect on TSE infectivity following prolonged digestion times. However, the majority of current TSE inactivation methods are aimed towards chemical and physical degradation procedures.

It is an object of the invention, therefore, to provide methods and means for effectively inactivating TSE infectious agents under conditions that can be readily applied to a variety of locations and situations. It is a further object of the invention to reduce the need for extreme conditions of very high temperature and harsh chemical denaturants in order to inactivate TSE agents located on or within TSE infected materials.

A first aspect of the invention provides a method for inactivating a TSE agent comprising exposing the TSE agent to a thermostable proteolytic enzyme.

The term transmissible spongiform encephalopathy (TSE) agent is intended to encompass all neurological diseases that are apparently transmitted via a pathogenic prion protein intermediate. Such TSEs typically include the human diseases Creutzfeld-Jacob disease (CJD), variant Creutzfeld-Jacob disease

(vCJD), Kuru, fatal familial insomnia and Gerstmann-Straussler-Scheinker syndrome. Non-human TSEs include bovine spongiform encephalopathy (BSE), scrapie, feline spongiform encephalopathy, chronic wasting disease, and transmissible mink encephalopathy. Given that vCJD is currently understood to be a human form of BSE, it is apparent that certain TSE agents are capable of crossing the species barrier and that novel TSEs from non-bovine sources could become evident in future.

The methods and compositions of the invention are suitable for the inactivation of TSE agent in apparatus and materials infected or suspected as being infected with TSE agent. In addition, the methods and compositions of the invention are suitably used in a prophylactic or precautionary mode, where definite knowledge of infection is uncertain. For example, the method of the invention can be easily incorporated into the standard sterilisation protocols used for preparation of surgical apparatus prior to use its use in surgical procedures.

The methods and compositions of the present invention are also suitably used for the inactivation of TSE agents in potentially contaminated clinical waste and culled animal material. At present, this waste material is incinerated at 1000°C, which requires specialised facilities and is expensive. It is an advantage of the present invention that the TSE inactivation procedure can occur at temperatures and in conditions that do not require highly specialised facilities and the prospects of complete inactivation of the TSE agent are comparable to the more energy intensive and expensive incineration procedures.

The proteolytic enzyme of the invention is typically a protease but can be suitably any biological polymeric molecule capable of catalysing cleavage of a polypeptide chain.

It is a feature of the invention that the proteolytic enzyme is a thermostable enzyme, that is, that it demonstrates optimal biological activity at temperatures in excess of the normal mammalian body temperature of 37°C. In embodiments of the invention the enzyme is thermally stable and biologically active, and inactivation is carried out, at temperatures equal to or greater than 40°C; preferably in the range of 50°C to 120°C; and more

preferably where the temperature is between 55°C and 85°C. In a specific embodiment of the invention the temperature is about 60°C. In a further specific embodiment the temperature is about 80°C.

5 Thermostable proteolytic enzymes suitable for use in the methods and compositions of the invention are obtainable from a number of sources such as thermophilic bacteria and archaea. In one embodiment of the present invention the thermostable proteolytic enzyme is isolated from thermophilic bacteria, hyperthermophilic bacteria and archaea. Suitable organisms for
10 extraction of proteolytic enzymes include *Thermotoga maritima*; *Thermotoga neopolitana*; *Thermotoga thermarum*; *Fervidobacterium islandicum*; *Fervidobacterium nodosum*; *Fervidobacterium pennivorans*; *Thermosiphon africanus*; *Aeropyrum pernix*; *Thermus flavus*; *pyrococcus spp.*; *Sulfolobus solfataricus*; *Desulfurococcus*; *Bacillus thermoproteolyticus*; *Bacillus stearo-*
15 *thermophilus*; *Bacillus sp. 11231*; *Bacillus sp. 11276*; *Bacillus sp. 11652*; *Bacillus sp. 12031*; *Thermus aquaticus*; *Thermus caldophilus*; *Thermus sp. 16132*; *Thermus sp. 15673*; and *Thermus sp. Rt41A*.

It is apparent to a person of skill in the art that the aforementioned organisms
20 are not the only sources of thermostable proteases. Indeed, some organisms that are not considered to be truly thermophilic can also express thermostable proteolytic enzymes. Such organisms are commonly termed thermodurable in that although they do not choose to live in conditions of high temperature, they can withstand high temperatures for limited periods. A number of *Bacillus*
25 species fall in the category of thermodurability and are known to produce thermostable subtilisin-type proteases.

The pH at which the inactivation is performed can range from acid to alkaline, but is typically in the region of pH 8-13, and preferably around pH 12.
30 Similarly, the thermostable protease is active in a pH range from acid to alkaline, but typically is optimally active in the region of pH 8-13, and preferably around pH 12.

In an example of the invention in use, the proteolytic enzyme is extracted from
35 a culture of the thermophilic bacteria or archaea. The culture is suitably maintained under the optimal conditions for the organism typically within a bioreactor. Thus, a continuous source of the organism can be maintained,

allowing proteolytic enzyme to be obtained whenever needed.

Alternatively, in an example of the invention in use described in more detail below, the gene encoding a thermostable proteolytic enzyme is isolated from the source organism, *Bacillus thermoproteolyticus*. The gene is used to generate a recombinant expression construct, typically a plasmid, which is transformed into a host organism, *Escherichia coli*. The transformed *E. coli* is grown in a bioreactor and when at an appropriate cell density the expression of the plasmid construct is initiated and the proteolytic enzyme harvested using standard methods. The recombinant expression route allows for the production of the proteolytic enzyme product under less extreme conditions of temperature than would be required for the original source organism.

The recombinant route is further advantageous in that it allows for the genetic manipulation of recombinant thermostable protease genes in order to increase thermal stability or biological activity or for some other purpose. Thus, the activity of a thermostable proteolytic enzyme can be readily optimised for use in the methods and compositions of the invention.

A second aspect of the invention provides for a method of sterilising apparatus, comprising the step of exposing the apparatus to a solution comprising a thermostable proteolytic enzyme.

The term "sterilising" is commonly understood to mean the procedure by which living organisms are removed from or killed in a substrate, such as a piece of equipment or a solution. In the present case the TSE agent, or prion, is not technically considered to be a living organism, in the sense that a bacterium or virus is, because it does not apparently contain any genetic material. However, the transmission of the TSE pathogenic agent between animals does result in disease. Thus, the term "sterilising" as used herein is applied to the procedure by which both pathogenic agents (such as TSE agents) and living organisms are removed from or killed in a substrate.

In a preferred embodiment, the method of the invention comprises maintaining the sterilising solution at a temperature below 100°C, and more preferably between 45°C and 85°C. The pH of the sterilising solution can range from acid to alkaline, but is typically in the region of pH 8-13, and preferably around

pH 12. Similarly, the thermostable protease is active in a pH range from acid to alkaline, but typically is optimally active in the region of pH 8-13, and preferably around pH 12.

5 In specific embodiments of the invention the sterilising solution is applied to the apparatus as a spray. The advantage of this mode of application is that, larger surface areas of apparatus, operating tables or even walls of rooms (for example in abattoirs) can be treated with the sterilising solution of the invention. Typically the solution will be heated to an optimal temperature, for
10 example between 60°C to 80°C, before being sprayed onto the surface that is to be sterilised, that surface being optionally heated in advance.

Alternatively, the apparatus is immersed in the sterilising solution for a predetermined period of time. Again, the temperature of the solution is
15 typically optimised prior to immersion of the contaminated apparatus. It is optional to include ultrasonication means in the immersion bath to enable ultrasonic cleaning to occur at the same time as treatment with the sterilising solution of the invention.

20 In a third aspect, the invention provides for a method of sterilising material, comprising exposing said material to a first solution comprising a thermostable proteolytic enzyme; and then exposing the apparatus to at least a second solution comprising a second thermostable proteolytic enzyme. In use, the material is typically apparatus, surgical or meat rendering equipment, or TSE
25 infected biological waste.

By dividing the sterilisation method into at least two, and optionally more, successive steps the conditions in each step can be optimised to ensure maximum inactivation of any TSE agent present. Thus, the temperatures
30 and/or pH of successive steps can be different. In specific embodiments of the invention the proteolytic enzymes in the first and second (and optionally more) solutions are the same, or are different.

In a fourth aspect the invention provides for a composition for inactivating a
35 TSE agent, comprising a thermostable proteolytic enzyme.

Typically, the composition of the invention further comprises a buffering

agent. In a specific embodiment of the invention the buffering agent has a pK_a of between 8 and 13. Alkaline buffers suitable for use in the method of the invention include 4-cyclohexylamino-1-butanedisulfonic acid (CABS) which has a pK_a of 10.7 at 25°C, and 3-cyclohexylamino-1-propanedisulfonic acid (CAPS) which has a pK_a of 10.4 at 25°C.

Alternatively, the composition of the invention comprises sufficient sodium hydroxide or other alkaline agent to adjust the pH of the composition to alkaline, more preferably around pH 12. Addition of 1M sodium hydroxide to the composition of the invention, using a pH probe calibrated using Universal standards, is sufficient to set the pH of the composition to around 12.

Further provided by the invention is apparatus for inactivating a TSE agent comprising:

- a. a chamber for receiving contaminated material;
- b. means for controlling the temperature of the chamber; and
- c. a thermostable proteolytic enzyme active at alkaline pH, located within the chamber, the chamber optionally containing a solution of the thermostable proteolytic enzyme at a temperature of 45°C to 85°C.

Further aspects of the invention provide for uses of the aforementioned compositions for the inactivation of TSE agents.

An advantage of the invention is its use in degrading TSE and similar agents, and it has been found in operation of particular embodiments of the invention that TSE-contaminated material has been successfully decontaminated using a combination of elevated temperature of around 50°C to 70°C and alkaline pH of around 9 to 12, with a thermostable, alkophilic proteolytic enzyme. Whilst it is on occasion possible to achieve some decontamination by extremely high temperature alone, it is of significant benefit to be able to inactivate TSE whilst avoiding extreme conditions, such as extremes of temperature, which lead to damage to the equipment being decontaminated

The methods and compositions of the inventions are described in more detail below and are illustrated by the accompanying drawings in which;

Fig. 1 shows the effect of temperature on a protease M digest of

mouse brain homogenate (mbh);

Fig. 2 shows the effect of pH on a protease M digest of mbh;

5

Fig. 3 shows a *Bacillus thermoproteolyticus* Rokko digest of mbh;

Fig. 4 shows the effect of sodium dodecyl sulfate (SDS) on Rokko digest of mbh;

10

Fig. 5 shows and SDS-PAGE of mbh;

Fig. 6 shows and immunoblot of mbh;

Fig. 7 shows a protease G, R and C digest of mbh;

15

Fig. 8 shows an immunoblot of the digest in Fig. 7.

EXAMPLES

VM Mouse Colony and Incubation with BSE (301V) agent

5 Studies on the inactivation of the TSE agent, BSE strain (301V), required the establishment of a mouse breeding colony for the generation of both uninfected and infectious brain homogenate (mbh) and its subsequent titration and bioassay. The VM mouse strain selected for use in the study was obtained from Dr David Taylor (Institute of Animal Health, Edinburgh). Six
10 pairs were introduced into a dedicated room within an animal facility. Mice were screened for their health status and a breeding programme initiated.

BSE (301V) infectious mouse brain (IAH, Edinburgh) was prepared for inoculation by crude homogenisation followed by passage of the brains
15 through increasingly fine gauge luer-locked needles (from 21G-27G) to and from a contained safety syringe into a closed septum-topped vial. This procedure was carried out in a validated safety cabinet within a containment level 3 (CL3) laboratory immediately prior to intracerebral inoculation of the VM mice. The anaesthetised (alphadolone/alphaxalone) mice were inoculated
20 intracerebrally via 26 gauge needles with 20ml of the mouse brain homogenate preparation. Forty-nine out of fifty mice survived this procedure. These were retained to allow incubation of the agent and the generation of the required quantity of high-titre infectious material.

25 Biomass Production

A wide variety of organisms were chosen for the production of biomass in order to provide as broad a selection of thermostable proteolytic enzymes as possible. Organisms selected ranged from those growing optimally at
30 moderately thermophilic temperatures (50°C) through to extremely thermophilic temperatures (100°C) and included members of both the Archaea and the Bacteria. Thermophiles were also selected to cover a wide range of growth pH, encompassing pH optima from pH2.5 to pH11.5. The majority of organisms were grown in batch culture, however, where the growth
35 requirements of the organism were particularly fastidious, continuous culture was used (Raven and Sharp (1997) Applied Microbial Physiology: A Practical Approach, Ch. 2, Eds. Stanbury and Rhodes, OUP pp.23-52). Depending on

the biomass yield of the organism being grown, batch culture volumes of between 20L and 120L were employed to achieve the desired amount of cell paste. The continuous culture system utilised either a 2L or 5L working volume gas lift bioreactor constructed entirely of glass and PTFE. More prolific organisms such as *Bacillus* spp., and *Thermus* spp., were pre-screened to select those with high levels of protease activity prior to their culture on a larger scale. A quick and sensitive fluorometric protease assay utilising microtitre plates was adopted for this purpose to permit high through-put screening (EnzChek™, Molecular Probes, Leiden, Netherlands).

Culture biomass was harvested by continuous centrifugation (Contifuge Stratos™, Kendro Laboratory Products, Bishop Stortford, UK) and stored at -80°C. Culture supernatants were concentrated with a 10KDa cut off tangential flow filter (Pall filtration, Portsmouth, UK). Proteins were precipitated with ammonium sulphate (90% saturation) and stored at -80°C.

Protein Purification

A rapid protease screening and purification technique was required in order to process all of the crude protein preparations after the biomass production stage. A dye-ligand affinity chromatography system was used for this purpose (PIKSI M™, Affinity Chromatography Ltd., Isle of Man, UK). Initially, each crude ammonium sulphate precipitate was dissolved in buffer and passed through a desalting column. Each sample was then loaded onto the PIKSI M test kit, which contained 10 different affinity ligands. Fractions were then assayed for protease activity to determine the most suitable matrix for purification of the protease, either by positive binding of the target molecule and then elution, or by negative binding of contaminants. The purification was then scaled up using the same affinity matrix in conjunction with an FPLC system (Amersham-Pharmacia Biotech, Amersham, UK). By combining this technique with the fluorometric protease assay, the rapid screening of many fractions could be undertaken.

The fluorometric protease assay utilises casein derivatives that are heavily labelled with green fluorescent BODIPY FL in which the conjugates' fluorescence is almost totally quenched. Protease catalysed hydrolysis releases the highly fluorescent label and the resultant fluorescence can be

measured on a fluorometric microplate reader (Labsystems Fluoroscan II™). The increase in fluorescence is proportional to protease activity and was compared with that of a standard protease (Protease X, Sigma-Aldrich, Poole, UK).

5

Protease Characterisation

10

A range of thermostable proteases were analysed (see Table 1). Direct characterisation of activity was carried out using the closest non-infectious analogue to BSE (301V) - infectious mouse brain homogenate available as substrate, i.e. normal VM mouse brain homogenate. Initial digests of total uninfected mouse brain homogenate (mbh) were performed over thirty minutes at 60°C and at pH7.0. The samples were then boiled under reducing conditions and analysed by SDS-PAGE on pre-cast NuPage 4 - 12% Bis-Tris gels (Novex™, San Diego, US). Gels were fixed using standard procedures and the proteins visualised using the Novex colloidal blue staining kit. The results showed both the levels of activity of the proteases against the mbh substrate under these conditions and also the level of purity of individual proteases. Protease concentrations used in the assays were based on total protein concentration, therefore, a wide range of activities was observed from limited to complete digestion.

15

20

25

30

Activity profiles showing the full effect of pH and temperature on mbh digestion were then produced for each enzyme. Several enzymes showed extensive activity over the complete range of conditions (pH 2 - 12 and 50 - 100°C). In general, however, complete digestion of mbh was only achieved over a narrower range indicative of the enzymes' pH and temperature optima. Figs. 1 and 2 show the activity profiles for a sample protease, *Bacillus* protease M. As can be seen the enzyme is moderately thermophilic and gives complete digestion at temperatures up to 70°C. Similarly the pH profile indicates a preference for neutral or alkaline conditions.

Table 1:

	Organism	Domain	Growth	Topt	pH opt
1	<i>Aeropyrum pernix</i>	Archaeon	Aerobe	95°C	7.0
2	<i>Alicyclobacillus acidocaldarius</i>	Bacterium	Aerobe	65°C	3.5
3	<i>Archaeoglobus fulgidus</i>	Archaeon	Anaerobe	85°C	6.5
4	<i>Bacillus caldotenax</i> BT1	Bacterium	Aerobe	65°C	7.0
5	<i>Bacillus pallidus</i>	Bacterium	Aerobe	65°C	9.0
6	<i>Bacillus stearothermophilus</i> L32-65	Bacterium	Aerobe	65°C	7.0
7	<i>Bacillus stearothermophilus</i> LUDA T57	Bacterium	Aerobe	65°C	7.0
8	<i>Bacillus thermoproteolyticus</i> Rokko	Bacterium	Aerobe	65°C	7.0
9	<i>Bacillus</i> sp. 11231	Bacterium	Aerobe	65°C	7.0
10	<i>Bacillus</i> sp. 11276	Bacterium	Aerobe	65°C	7.0
11	<i>Bacillus</i> sp. 11652	Bacterium	Aerobe	65°C	7.0
12	<i>Bacillus</i> sp. 12031	Bacterium	Aerobe	65°C	7.0
13	<i>Desulfurococcus</i> sp.	Archaeon	Anaerobe	85°C	6.5
14	<i>Fervidobacterium pennivorans</i>	Bacterium	Anaerobe	70°C	8.5
15	<i>Hyperthermus butylicus</i>	Archaeon	Anaerobe	95°C	6.5
16	<i>Pyrococcus furiosus</i>	Archaeon	Anaerobe	95°C	7.5
17	<i>Pyrococcus horikoshii</i>	Archaeon	Anaerobe	95°C	7.0
18	<i>Sulfolobus acidocaldarius</i> 98-3	Archaeon	Aerobe	75°C	2.5
19	<i>Sulfolobus hakonensis</i>	Archaeon	Aerobe	75°C	2.5
20	<i>Sulfolobus solfataricus</i> P1	Archaeon	Aerobe	75°C	2.5
21	<i>Sulfolobus solfataricus</i> P2	Archaeon	Aerobe	75°C	2.5
22	<i>Thermobrachium celere</i>	Bacterium	Anaerobe	65°C	8.5
23	<i>Thermococcus fumicolans</i>	Archaeon	Anaerobe	85°C	6.5
24	<i>Thermus caldophilus</i> GK24	Bacterium	Aerobe	70°C	8.0
25	<i>Thermus aquaticus</i> YT1	Bacterium	Aerobe	70°C	8.0
26	<i>Thermus</i> sp. 16132	Bacterium	Aerobe	70°C	8.0
27	<i>Thermus</i> sp. 15673	Bacterium	Aerobe	70°C	8.0
28	<i>Thermus</i> sp. Rt41A	Bacterium	Aerobe	70°C	8.0

Protease Testing

Several enzymes did not give complete digestion of mbh even when incubation times were increased to 24 hours. This may have simply been due to low protease activity, however, several highly purified and concentrated enzymes still only produced partial digests. In these reactions little enzyme-substrate interaction appeared to be occurring. It was considered that this could be due to non-proteinaceous material, e.g. lipids, surrounding the substrate and preventing interaction, however, pre-treatment with lipases and other enzymes had no observable effect (data not shown). A second possibility considered was the effect of repulsive surface charges between proteases and the substrate. One enzyme in particular, purified from *Bacillus thermoproteolyticus*

Rokko, consistently produced poor digestion of mbh even at high concentrations. This enzyme was known to remain active in the presence of high levels of detergents, therefore, digests were performed with the addition of SDS in an attempt to overcome this effect. Figures 3 and 4 show

5 *B.thermoproteolyticus* Rokko digests of mbh in the absence and presence of SDS. Figure 3 clearly indicates that under standard conditions there is little difference in digestion even as the protease concentration is increased to 20mg.ml⁻¹. On addition of SDS (lanes 2-5), no protein bands, except the

10 protease itself, are visible indicating a complete digest. Tested proteases could, therefore, be divided simply into three categories: (i) those able to give total mbh digestion in the absence of detergent, (ii) those able to give total mbh digestion in the presence of SDS and (iii) those unable to digest the substrate fully. Proteases in categories (i) and (ii) were selected for immediate further study, while those in category (iii) were either rejected or assumed to be

15 required in greater quantity and/or higher purity.

Western Blotting of Mouse Brain Homogenate

Mbh proteins were transferred onto nitrocellulose and blocked overnight in PBS-Tween (PBST) + 3% skimmed milk powder. The membrane was washed (x3 in

20 PBST) and incubated for 1 hour with 6H4 anti-human recombinant PrP monoclonal antibody (Prionics, Zurich, Switzerland). After a second washing step, anti-mouse HRP-conjugate was added and the membrane incubated for 1 hour. Washing was repeated and the antibody reaction visualised by addition of TMB (Harlow and Lane

25 (1988), Antibodies: A Laboratory Manual, Cold Spring Harbor Press).

Figures 5 and 6 show an SDS-PAGE and an immunoblot of undigested mbh respectively. Although there is some non-specific background, dark bands indicating the presence of mouse prion can clearly be seen at the expected

30 molecular weight (~33-35kDa). Distinct bands are also visible at ~66-70kDa, which may correspond to prion dimers previously reported (Safar *et al.* (1990) Proc. Natl. Acad. Sci. USA, 87:pp6373-6377).

Western transfer and immunoblotting were used to confirm that no

35 immunoreactive fragment of mouse PrP^C remained after digestion. Figure 7 shows mbh digested to completion with three closely related thermostable

proteolytic enzymes (Proteases G, R and C), as assessed by SDS-PAGE. In the corresponding immunoblot (Figure 8) only 2 bands are visible, both with an apparent MW of ~23kDa. The strong band in lane 9 corresponds to recombinant mouse PrP (+ve control), whilst the weak band in lane 4 appears to be due to a slight reaction with the heavily loaded enzyme preparation. In this case, complete loss of PrP^C immunoreactivity is still apparent since no band is observable in lane 3.

Preparation and Titration of Infectious Mouse Brain Homogenate

Eighty days post challenge onward, mice were subject to daily clinical scoring to detect clinically affected mice as early as possible. A single mouse died of an unknown cause prior to this period. The remainder exhibited the expected disease progression and were sacrificed between 110 and 130 days post challenge. The brains were removed aseptically and stored frozen until required. Forty-eight BSE (301V) infectious VM mouse brains were homogenised in four volumes of PBS within a contained homogeniser then passed sequentially through increasingly fine gauge needles (21G to 26G) until free flowing. A sample with a further 2-fold dilution (1:9 mouse brain: PBS) was prepared for titration of infectivity. Over 800 x 0.1ml aliquots of BSE (301V) infectious mouse brain homogenate were prepared. These procedures were again carried out under rigorous class III containment, including the wearing of positive pressure respirators.

Groups of 25 eight week old VM mice received titration doses of the infectious mouse brain homogenate preparation at 10 fold dilutions from 10^{-1} – 10^{-8} . A further group of 25 mice were challenged with uninfected mouse brain homogenate as a control. All mice were inoculated under anaesthetic using 26G x 3/8" needles with plastic sleeve guards cut off 2mm below bevel in a Class 2 cabinet with the use of an injection guard. The mice were then left to incubate the BSE (301V) agent for extended periods some in excess of a year. The initial titre of the infectious mouse brain homogenate preparation was established retrospectively once all incubations (clinical monitoring at 80 days onwards) were complete.

CLAIMS

1. A method for inactivating a transmissible spongiform encephalopathy (TSE) agent comprising exposing the TSE agent to a thermostable proteolytic enzyme.
2. A method according to claim 1, comprising exposing the TSE agent to the thermostable protease at a temperature that is equal to or greater than 40°C.
3. A method according to claim 2, wherein the temperature is between 50°C and 120°C.
4. A method according to claim 3, wherein the temperature is between 55°C and 85°C.
5. A method according to claim 4, wherein the temperature is about 60°C.
6. A method according to claim 4, wherein the temperature is about 80°C.
7. A method according to any previous claim wherein the pH is neutral (pH 7.0).
8. A method according to claims 1-6, wherein the pH is acidic.
9. A method according to claims 1-6, wherein the pH is alkaline.
10. A method according to claim 9, wherein the pH is between 8 and 13.
11. A method according to claim 9, wherein the pH is about 12.
12. A method according to any previous claim wherein the TSE agent is a prion.

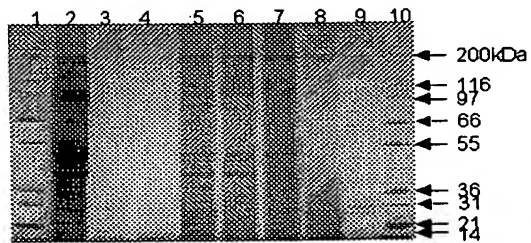
13. A method according to any previous claim, wherein the TSE agent is selected from the group consisting of Creutzfeld-Jacob disease; variant Creutzfeld-Jacob disease; Kuru; fatal familial insomnia; Gerstmann-Straussler-Scheinker syndrome; bovine spongiform encephalopathy; scrapie; feline spongiform encephalopathy; chronic wasting disease; and transmissible mink encephalopathy.
14. A method according to any previous claim wherein the thermostable proteolytic enzyme is obtained from a thermophilic organism.
15. A method according to claim 14 wherein the thermophilic organism is selected from the group consisting of archaea; hyperthermophilic bacteria and thermophilic bacteria.
16. A method according to claim 15 wherein the thermophilic organism is selected from the group consisting of *Thermotoga maritima*; *Thermotoga neopolitana*; *Thermotoga thermarum*; *Fervidobacterium islandicum*; *Fervidobacterium nodosum*; *Fervidobacterium pennivorans*; *Thermosipho africanus*; *Aeropyrum pernix*; *Thermus flavus*; *pyrococcus spp.*; *Sulfolobus solfataricus*; *Desulfurococcus*; *Bacillus thermoproteolyticus*; *Bacillus stearo-thermophilus*; *Bacillus sp. 11231*; *Bacillus sp. 11276*; *Bacillus sp. 11652*; *Bacillus sp. 12031*; *Thermus aquaticus*; *Thermus caldophilus*; *Thermus sp. 16132*; *Thermus sp. 15673*; and *Thermus sp. Rt41A*.
17. A method of sterilising apparatus comprising exposing said apparatus to a solution comprising a thermostable proteolytic enzyme.
18. A method according to claim 17, wherein the solution is maintained at a temperature below 100°C.
19. A method according to claim 17, wherein the solution is maintained at a temperature of between 45°C and 85°C.

20. A method according to any of claims 17-19, wherein the solution has a neutral pH.
21. A method according to any of claims 17-19, wherein the solution has an acidic pH.
22. A method according to any of claims 17-19, wherein the solution has an alkaline pH.
23. A method according to claim 22, wherein the solution has a pH of between 8 and 13.
24. A method according to any of claims 17-23, wherein the thermostable proteolytic enzyme is obtained from a thermophilic organism.
25. A method according to claim 24 wherein the thermophilic organism is selected from the group consisting of archaea; hyperthermophilic bacteria and thermophilic bacteria.
26. A method according to any of claims 17-25, wherein the solution is applied to the apparatus as a spray.
27. A method according to any of claims 17-25, wherein the apparatus is immersed in the solution.
28. A method of sterilising apparatus, comprising exposing said apparatus to a first solution comprising a thermostable proteolytic enzyme; and then exposing the apparatus to at least a second solution comprising a second thermostable proteolytic enzyme.
29. A method according to claim 28, wherein the first and second proteolytic enzymes are the same.
30. A method according to claim 28, wherein the first proteolytic enzyme is different to the second proteolytic enzyme.

31. A method according to any of claims 28-30, wherein the pH of the first solution is different to the pH of the second solution.
32. A method according to any of claims 28-31, wherein the temperature of the first solution is different to the temperature of the second solution.
33. A composition for inactivating a TSE agent, comprising a thermostable proteolytic enzyme.
34. A composition according to claim 33, further comprising a buffering agent.
35. A composition according to claim 34, wherein the buffering agent has a pK_a of between 8 and 13.
36. A composition according to claim 33, further comprising sufficient sodium hydroxide to set the pH of the composition to around 12.
37. A composition according to claims 33-36, further comprising a detergent compound.
38. A composition according to claim 37, wherein the detergent is sodium dodecyl sulphate (SDS).
39. A composition according to any of claims 33-38, wherein the thermostable proteolytic enzyme is obtained from a thermophilic organism.
40. A composition according to claim 39 wherein the thermophilic organism is selected from the group consisting of archaea; hyperthermophilic bacteria and thermophilic bacteria.
41. Use of a composition according to claims 33-40 for sterilising material contaminated with a TSE agent.
42. Apparatus for inactivating a TSE agent comprising:

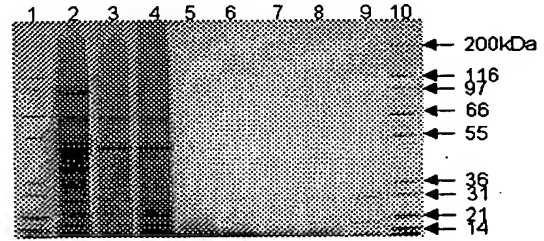
- a. a chamber for receiving contaminated material;
 - b. means for controlling the temperature of the chamber; and
 - c. a thermostable proteolytic enzyme active at alkaline pH, located within the chamber.
43. Apparatus according to claim 42 comprising a solution of the thermostable proteolytic enzyme at a temperature of 45°C to 85°C.

Fig. 1

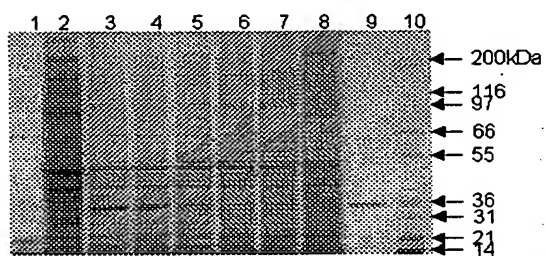


Lanes 1 & 10, marker proteins; lane 2 untreated mbh; lane 3, 50°C; lane 4, 60°C; lane 5, 70°C; lane 6, 80°C; lane 7, 90°C; lane 8, 100°C; lane 9, Protease M.

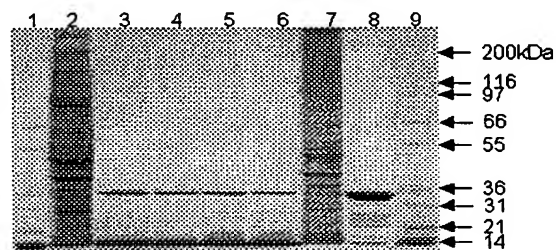
Fig. 2



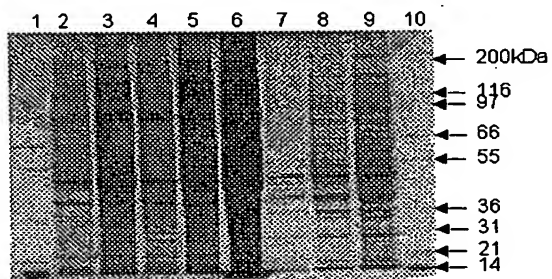
Lanes 1 & 10, marker proteins; lane 2 untreated mbh; lane 3, pH2; lane 4, pH4; lane 5, pH6; lane 6, pH8; lane 7, pH10; lane 8, pH12; lane 9, Protease M.

Fig. 3

Lanes 1 & 10, marker proteins;
lane 2 untreated mbh; lanes 3 - 8,
Rokko digest (20mg.ml⁻¹ -
0.1mg.ml⁻¹), lane 9, Rokko
(1mg.ml⁻¹).

Fig. 4

Lanes 1 & 9, marker proteins; lane 2
untreated mbh; lane 3, 2% SDS; lane
4, 1% SDS; lane 5, 0.5% SDS; lane 6,
0.25% SDS; lane 7, mbh + 2% SDS;
lane 8, Rokko (20mg.ml⁻¹).

Fig. 5

Lanes 1 & 10, marker proteins; lanes 2 & 3, mbh; lanes 4 - 6, mbh pellet; lanes 7 - 9, mbh supernatant.

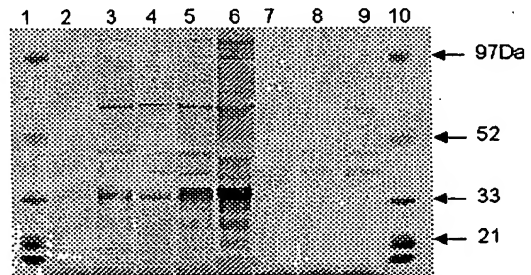
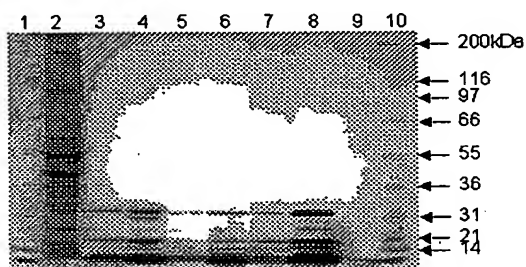
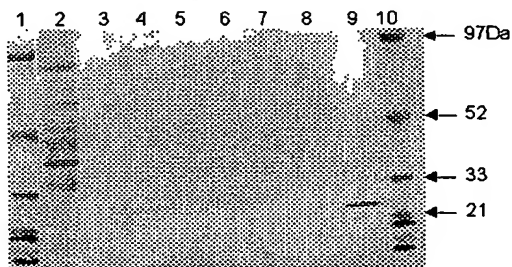
Fig. 6

Fig.7**Fig.8**

Lanes 1 & 10, marker proteins; lane 2, untreated mbh; lane 3, Protease G digest; lane 4, Protease G; lane 5, Protease R digest; lane 6, Protease R; lane 7, Protease C digest; lane 8, Protease C; lane 9, rec. mouse PrP.

